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Binding of random copolymers of three amino acids to class II MHC molecules

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Abstract

Copolymer 1 [Cop 1, poly(Y,E,A,K)] is a random synthetic amino acid copolymer of L-tyrosine, L-glutamic acid, L-alanine and L-lysine, effective both in suppression of experimental allergic encephalomyelitis and in the treatment of relapsing forms of multiple sclerosis. Cop 1 binds promiscuously and very efficiently to purified human HLA-DR molecules within the peptide-binding groove. In the present study the binding of copolymers composed of three of the four amino acids found in poly(Y,E,A,K) to purified class II MHC molecules was examined. Poly(Y,A,K) and poly(Y,E,A,K) bound to purified human HLA-DR1 or -DR4 molecules with affinity higher than poly(Y,E,A), poly(E,A,K) or poly(Y,E,K), whereas poly(Y,E,A,K) and poly(E,A,K) were the better binders of HLA-DR2 molecules. On the other hand, poly(Y,E,A) and poly(Y,A,K) inhibited the binding of biotinylated poly(Y,E,A,K) to these molecules 10-fold more efficiently than poly(Y,E,K). Finally, poly(Y,E,A), poly(Y,A,K) and poly(E,A,K) were cross-reactive with poly(Y,E,A,K) using YEAK-specific T cell lines and clones of mouse or human origin.

Introduction

Copolymer 1 [Cop 1, poly(Y,E,A,K)] is a synthetic amino acid copolymer effective both in suppression of experimental allergic encephalomyelitis (EAE) (1–5) and in the treatment of relapsing forms of multiple sclerosis (MS) (6,7). The activity of Cop 1 in EAE and MS appears to involve, as the first step, binding to class II MHC molecules, following which two pathways may be activated: (i) induction of antigen-specific suppressor cells (4,8) and (ii) competition with myelin antigens for activation of specific effector T cells (9,10). Cop 1 bound both to living antigen-presenting cells (APC) of various HLA haplotypes and to mouse APC (11), with no proteolysis required (12). Moreover, it both competed with and displaced the autoantigens myelin basic protein (MBP), proteolipid protein (PLP) and myelin oligodendrocyte glycoprotein (MOG) from their binding sites on APC (13,14). More specifically, Cop 1 bound to purified human HLA-DR molecules in a peptide antigen-specific manner, i.e. within the peptide-binding groove, and inhibited the binding of HA 306–318, a high-affinity peptide epitope of influenza virus, to both HLA-DR1 (DRB1*0101) and DR4 (DRB1*0401) molecules, and the binding of MBP 85–99 to HLA-DR2 (DRB1*1501) (15). Cop 1-related copolymers in which E was replaced by D [poly(Y,D,A,K)], poly(E,A,K) (2) and a copolymer in which Y was replaced by W [poly(W,E,A,K), Cop 4] (16) were all active in suppressing EAE. On the other hand, the acidic copolymer poly(Y,E,A) was inactive. In order to further characterize the requirements for efficient binding of Cop 1 to class II MHC molecules, in the present study four random copolymers composed of three of the four amino acids of Cop 1 [poly(E,A,K), poly(Y,E,A), poly(Y,A,K) and poly(Y,E,K)] were examined for binding to several class II MHC molecules of human origin.

Methods

Cell lines and antibodies

Homozygous Epstein–Barr virus-transformed human B lymphocyte lines used for immunoaffinity purification of HLA-DR1, -DR2 and -DR4 molecules were LG-2 (DRB1*0101), MGAR (DRB1*1501) and Priess (DRB1*0401/DRB4*0101) respectively. Cells were grown in RPMI 1640 supplemented

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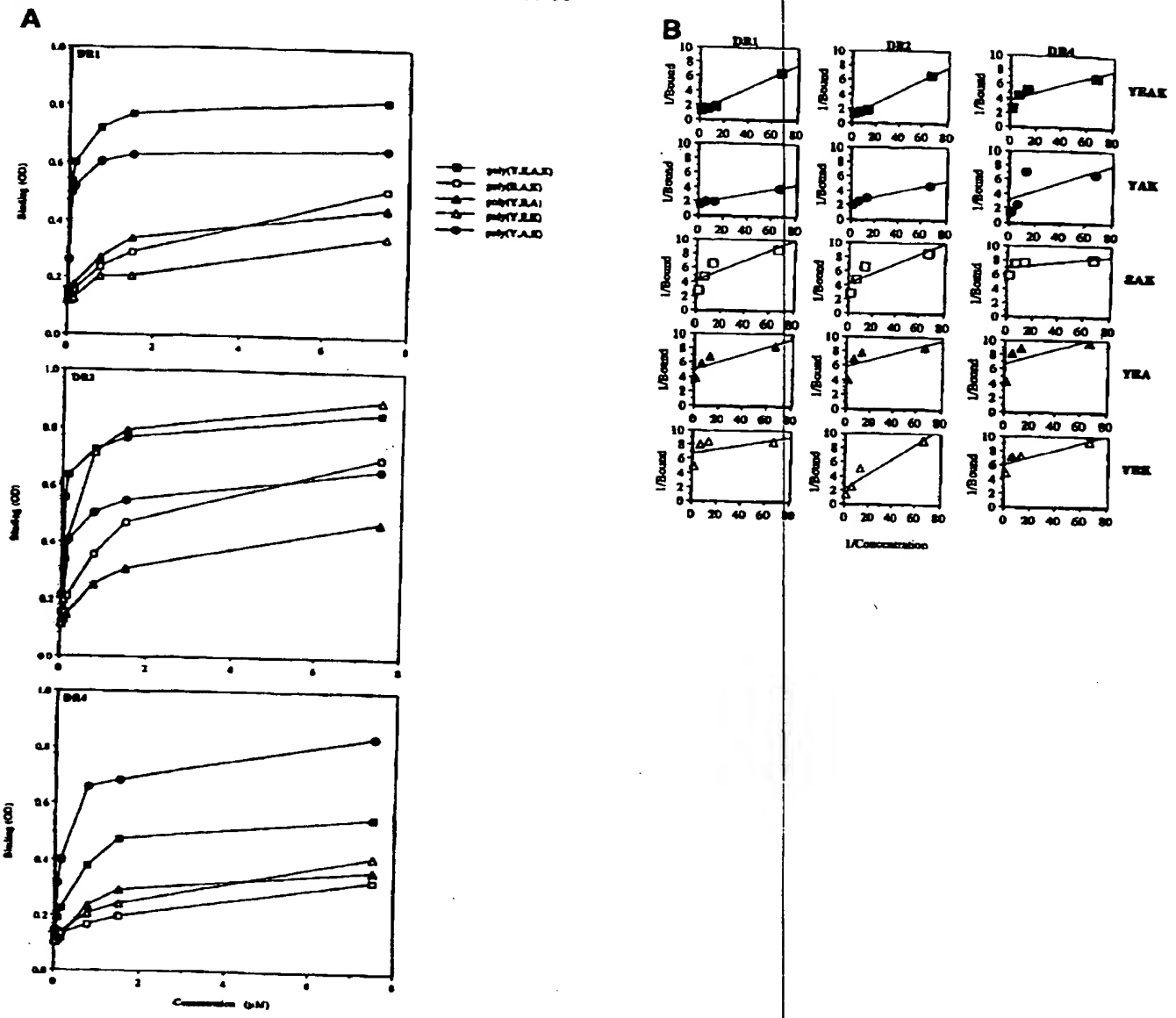

Fig. 1. (A) Binding of poly(Y,E,A,K), poly(E,A,K), poly(Y,E,A), poly(Y,E,K) and poly(Y,A,K) to HLA-DR proteins. Detergent-solubiliz d HLA-DR1, -DR2 and -DR4 molecules (0.5 µg/sample) were incubated in duplicate with a range of concentrations of biotinylated poly(Y,E,A,K) (mol. wt 5800), poly(E,A,K) (mol. wt 8850), poly(Y,E,A) (mol. wt 7600), poly(Y,A,K) (mol. wt 20,000) and poly(Y,E,K) (mol. wt 11,050) at pH 5.0 for 40 h at 37°C. Peptide–class II MHC protein complexes were captured with LB3.1 and biotin-peptide binding was measured. (B) Lineweaver–Burk plots of the binding data.

with 10% FCS, 2 mM glutamine, 50 units/ml penicillin G and 50 µg/ml streptomycin in roller bottles and stored as pellets at −80°C. The anti-DR hybridoma LB3.1 (IgG2b) (17) was grown in serum-free medium (Macrophage-SFM; Gibco/BRL, Grand Island, NY).

Protein purification

Immunoaffinity purification of HLA-DR1, -DR2 and -DR4 molecules was performed as previously reported (18), with minor modifications. Briefly, detergent-soluble membrane preparations from LG-2, MGAR and Priess cells were passed at a flow rate of ~11 ml/h through a series of columns in the following sequence: Sepharose CL-6B (30 ml), normal mouse serum-Affi-gel 10 (10 ml), Protein A–Sepharose CL-4B (5 ml) and LB3.1–Protein A–Sepharose CL-4B (5 ml). DR2a (DRB5*0101) and DRw53 (DRB4*0101), the products f DR genes linked to the DRB1 alleles, were not removed from the MGAR and Priess lysates before passage through the LB3.1 immunoaffinity column, and contaminate the DR2 and DR4 preparations in th amount of 5–10%. All the subsequent

Table 1. Affinity of the binding of synthetic copolymers to purified human HLA-DR1, -DR2 and -DR4 molecules

Copolymer[a]	DR1[b]		DR2		DR4	
	K_d[c]	IC_{50}[d]	K_d	IC_{50}	K_d	IC_{50}
Poly(Y,E,A,K)	7.4	8.8	8.2	10.1	1.5	10.8
Poly(Y,A,K)	2.0	3.3	1.7	2.7	2.0	6.5
Poly(E,A,K)	0.5	–[e]	1.7	–[e]	0.3	[e]
Poly(Y,E,A)	1.0	1.3	0.8	9.5	0.8	1.0
[tbPoly(Y,E,K)	0.4	43.0	5.0	25.4	0.8	43.0

[a]Poly(Y,E,A,K) with average mol. wt of 5800; poly(Y,A,K), mol. wt 20,000; poly(E,A,K), mol. wt 8850; poly(Y,E,A), mol. wt 7600 and poly(Y,E,K), mol. wt 11,050 were incubated at a range of concentrations with purified HLA-DR1, -DR2 and -DR4 molecules at pH 5.0 followed by capture with class II-specific mAb and peptide detection with alkaline phosphatase–streptavidin.

[b]Detergent-soluble HLA-DR1, -DR2 and -DR4 molecules were purified as described in Methods.

[c]The dissociation constant at equilibrium calculated from the slope of the double-reciprocal plot (Fig. 1B) and expressed as $\times 10^{-8}$ M.

[d]Inhibitory concentration giving 50% inhibition calculated based on the competitive binding assays (Fig. 2) and are expressed as $\times 10^{-6}$ M.

[e]IC_{50} values for poly(E,A,K) could not be determined exactly, but were <1000 μM (see Fig. 2).

steps were as previously described (18). The eluate was dialyzed against 0.1% deoxycholate, 10 mM Tris–HCl, pH 8 and concentrated on a Centriprep 30 membrane (Amicon, Beverley, MA). Protein concentrations were determined by bicinchoninic acid assay (Pierce, Rockville, IL).

Peptides and copolymers

Cop 1, poly(Y,E,A,K), is a synthetic random copolymer prepared by polymerization of the *N*-carboxyanhydrides of L-tyrosine, γ-benzyl-L-glutamate, L-alanine and ε,*N*-trifluoroacetyl-L-lysine, followed by removal of the blocking groups (1) (the end product is a mixture of acetate salts of random polypeptides). Poly(E,A,K), poly(Y,E,A), poly(Y,A,K) and poly(Y,E,K) were synthesized similarly. Cop 1, poly(Y,E,A,K), batch 55495, in the molar ratio of 1 Y:1.5 E:4.8 A:3.7 K, with an average mol. wt of 5800 or batch 52596, 1 Y:1.5 E:4.3 A:3.3 K, mol. wt 8150; poly(E,A,K), batch SD-1689, 1.5 E:4.7 A:3.6 K, mol. wt 8850; poly(Y,E,A), batch SD-1690, 1 Y:1.5 E:4.8 A, mol. wt 7600; poly(Y,A,K), batch SD-1691, 1 Y:5.3 A:3.5 K, mol. wt 20,000 and poly(Y,E,K), batch SD-1697, 1 Y:1.6 E:3.6 K, mol. wt 11,050 were synthesized by Drs B. Dolitzky and D. Salner of Teva Pharmaceutical Industries (Petach Tikva, Israel). Peptides were synthesized using solid-phase techniques (19) on an Applied Biosystems Peptide Synthesizer and purified by reversed-phase HPLC. The peptides used were HA 306–318, PKYVKQNTLKLAT, mol. wt 1718 and MBP 84–102, DENPVVHFFKNIVTPRTPP, mol. wt 2529.

Peptide labeling

Biotinylation of the various copolymers was performed with excess *N*-hydroxysuccinimide–biotin (Sigma, St. Louis, MO) in dimethyl sulfoxide as described (11). Unreacted biotin was removed by dialysis (Spectra/Por membrane MWCO 500; Spectrum Medical Industries, Laguna Hills, CA).

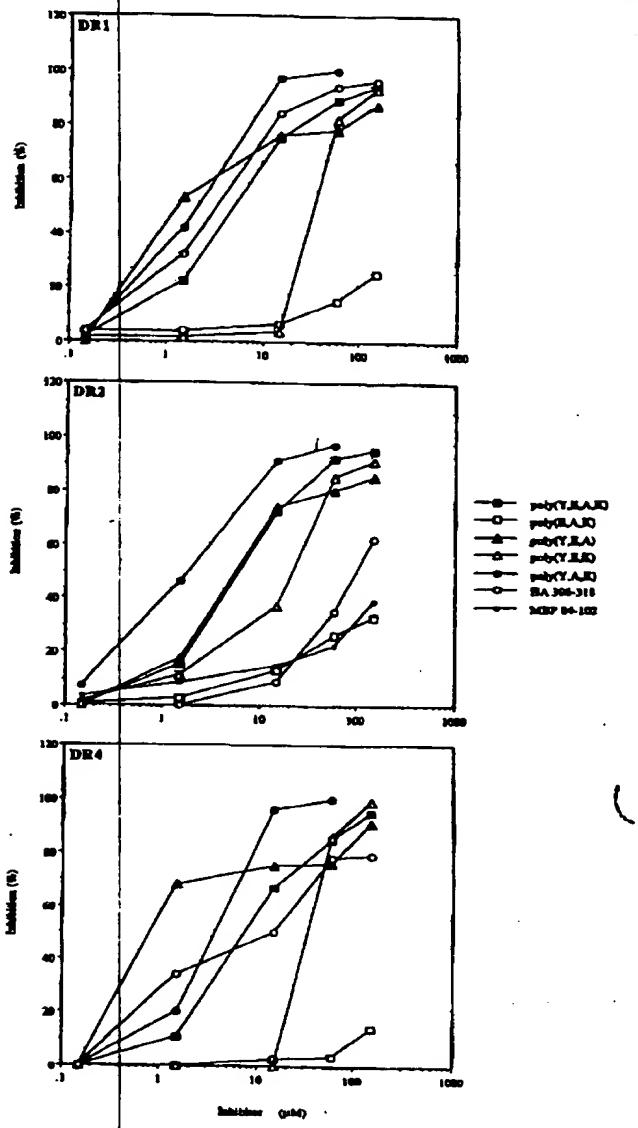

Fig. 2. Inhibition of poly(Y,E,A,K) binding to HLA-DR molecules by different competitors. Purified HLA-DR1, -DR2 or -DR4 were incubated with biotinylated poly(Y,E,A,K) (mol. wt 8150) (1.5 μM) alone or in the presence of unlabeled poly(Y,E,A,K), poly(E,A,K), poly(Y,E,A), poly(Y,E,K), poly(Y,A,K), MBP 84–102 or HA 306–318, at a range of concentrations. All incubations were carried out in duplicat at pH 5.0 for 40 h at 37°C. Specific binding is expressed as percentage of inhibition using the formula: percentage of inhibition = 100% – [(signal with competitor – background)/signal without competitor – background)×100].

Assays for peptide binding to class II MHC proteins

Solutions. The solutions used in this assay are the following: binding buffer = 20 mM 2-[*N*-morpholino]ethanesulfonic acid, 1% *n*-octyl β-D-glycopyranoside, 140 mM NaCl, 0.05% NaN_3,

pH 5.0, unless otherwise specified; PBS = 150 mM sodium chloride, 7.5 mM sodium phosphate, dibasic, 2.5 mM sodium phosphate, monobasic, pH 7.2; TBS = 137 mM sodium chloride, 25 mM Tris pH 8.0, 2.7 mM potassium chloride; TTBS = TBS plus 0.05% Tween-20.

Microtiter assay plate preparation. Ninety-six-well microtiter immunoassay plates (PRO-BIND; Falcon, Lincoln Park, NJ) were coated with 1 µg/well affinity-purified LB3.1 mAb in PBS (100 µl total) for 18 h at 4°C. The wells were then blocked with TBS/3% BSA for 1 h at 37°C and washed 3 times with TTBS. Before sample addition, 50 µl of TBS/1% BSA was added to each well.

Binding reactions. Detergent-solubilized HLA-DR1, -DR2 and -DR4 molecules (0.5 µg/sample) were incubated with biotinylated peptides at various concentrations for 40 h at 37°C in 50 µl of the binding buffer, and transferred to prepared microtiter assay plates and incubated for 1 h at 37°C for capture of peptide–class II complexes.

Inhibition reactions. Biotinylated copolymers at a final concentration of 1.5 µM in 50 µl of the binding buffer were co-incubated with the unlabeled copolymers as well as the peptides HA 306–318 or MBP 84–102, used as inhibitors, and HLA-DR molecules for 40 h at 37°C.

Detection of class II–peptide complexes. Bound peptide–biotin was detected using streptavidin-conjugated alkaline phosphatase as follows. Plates were washed 3 times with TTBS and incubated with 100 µl of streptavidin-conjugated alkaline phosphatase (1:3000; BioRad, Richmond, CA) for 1 h at 37°C, followed by addition of *p*-nitrophenyl phosphate in triethanolamine buffer (BioRad). The absorbance at 410 nm was monitored by a microplate reader (Dynatech MR4000).

T cell lines and clones

Cop 1-specific T cell lines and clones were: LN-1, LN-3 clones (derived from lymph nodes of (SJL/J×BALB/c)F_1 mice injected with Cop 1 in complete Freund's adjuvant); S-3 line and S-22-5 clone (from spleens of (SJL/J×BALB/c)F_1 mice injected with Cop 1 in incomplete Freund's adjuvant), according to Aharoni *et al.* (8,20), and C-14 and C-52 clones (derived from a DR7,w11 donor with MS), according to Teitelbaum *et al.* (10).

Proliferation assay

Cross-reactivity of the various polymers with Cop 1 was evaluated by their ability to stimulate Cop 1-specific T cell lines and clones of mouse and human origin. T cells (1.5×10^4) were cultured in triplicates with irradiated mouse spleen cells (5×10^5) or with human Epstein–Barr virus-transformed B cells (5×10^4) and with the indicated antigens in a final volume of 0.2 ml in 10% FCS culture medium. At the end of 48 h incubation, cultures were pulsed with 1 µCi [^{3}H]thymidine and harvested 6–12 h later. The variations of triplicates from their mean were <20%.

IL-4 secretion

Secretion of IL-4 by T cell lines and clones in response to the various polymers was valuat d by the ability of their cultur supernatant to support the proliferation of the IL-4-dependent line CT4-S. T cells (1.5×10^4) were incubated with the indicated antigen presented on irradiated spleen cells (5×10^5). Supernatants (50 µl) were collected at the end of 48 h and incubated with the CT4-S indicator cells (1×10^4/well) at a 1:1 dilution to a final volume of 0.1 ml for 48 h and then labeled with 1 µCi-[^{3}H]thymidine for 16 h. The variations of triplicates from their mean were <20%. For each copolymer the results obtained correspond to the conc ntration which gave maximal cross-reactive response, i.e. poly(Y,E,A,K), poly(E,A,K), poly(Y,E,A) and poly(Y,E,K) at 10 µg/well, and poly(Y,A,K) at 2.5 µg/well.

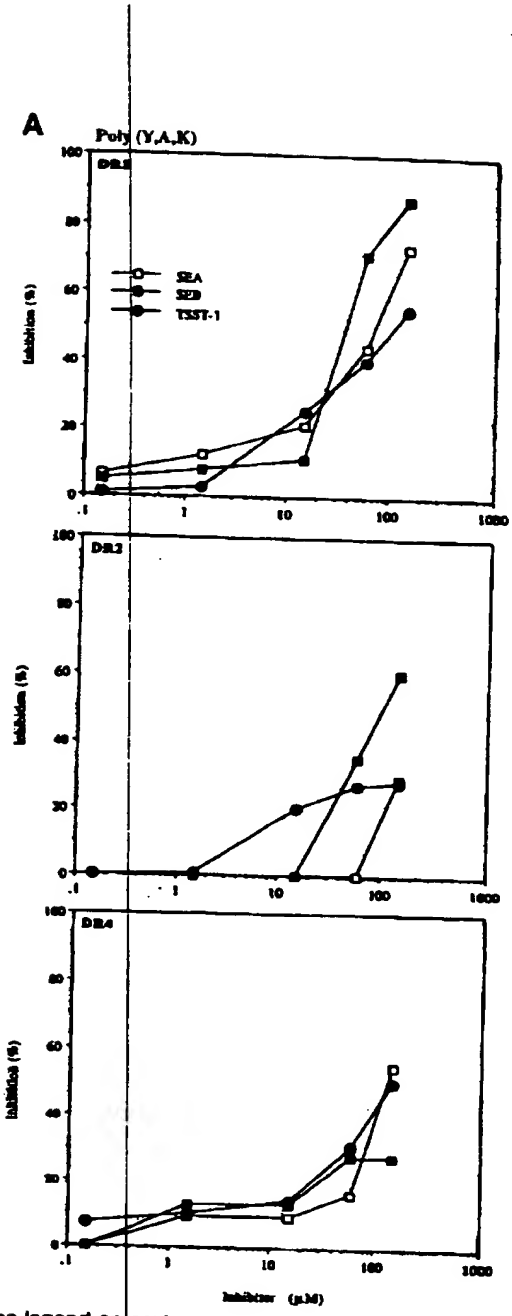

Fig. 3. See legend on next page.

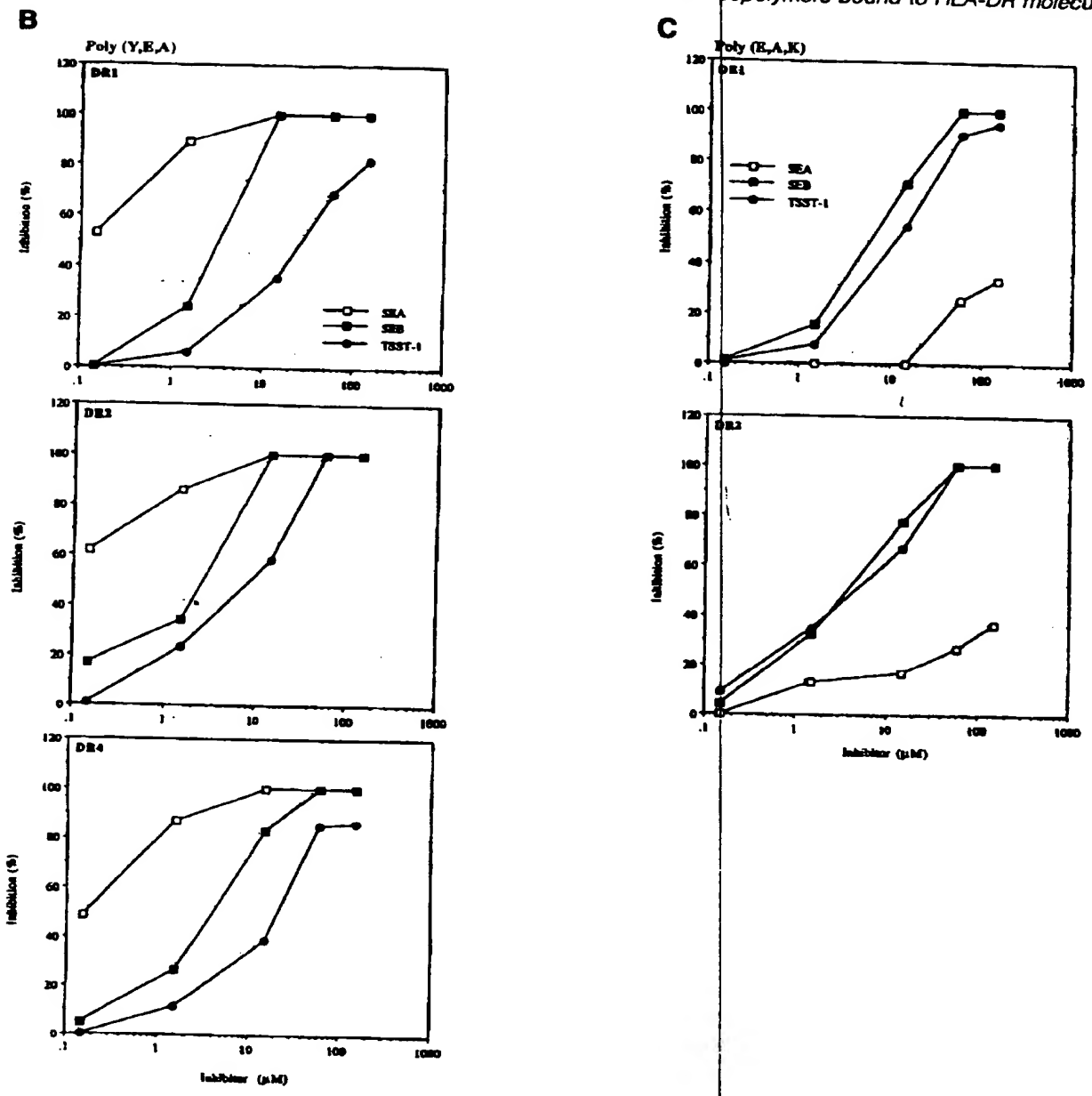

Fig. 3. Inhibition of binding of poly(Y,A,K) (A), poly(Y,E,A) (B) and poly(E,A,K) (C) to HLA-DR molecules by superantigens. Purified HLA-DR1, -D or -DR4 were incubated with biotinylated poly(Y,A,K), poly(Y,E,A) or poly(E,A,K) (1.5 μM) alone or in the presence of unlabeled SEA, SEB or TS 1 at a range of concentrations. All incubations were carried out in duplicate at pH 5.0 for 40 h at 37°C. For methods see legend for Fig. 2.

Results

Binding of the copolymers of three amino acids to purified HLA-DR molecules

Detergent-soluble HLA-DR1, -DR2 and -DR4 proteins were purified from homozygous Epstein–Barr virus-transformed B cell lines LG-2 (DRB1*0101), MGAR (DRB1*1501) and Priess (DRB1*0401) respectively as described previously (15). Three different preparations of Cop 1 had bound to these molecules with high affinity (15). To determine the affinity of the copol mers of three amino acids for HLA-DR proteins, binding assa were carried out with biotinylated poly(E,A,K), poly(Y,E,A poly(Y,A,K) and poly(Y,E,K), and compared to poly(Y,E,A,K (Cop 1). The copolymers were incubated at a range concentrations with purified HLA-DR1, -DR2 and -DR4 mole ules at pH 5.0 followed by capture with class II-specific mA and peptide detection with alkaline phosphatase–streptavidi The binding of poly(Y,A,K) and Cop 1 to detergent-solub

HLA-DR1 and -DR4 molecules was better than that of poly(E,A,K), poly(Y,E,A) or poly(Y,E,K), whereas poly(Y,E,K) and Cop 1 bound better than the other copolymers to HLA-DR2, based on the saturation binding curves (Fig. 1A), and on K_d values, calculated from the double-reciprocal plots of the binding data (Fig. 1B and Table 1). Poly (Y, A, K) bound to HLA-DR4 particularly efficiently. It is also obvious from these data that the different copolymers bound to the purified HLA-DR proteins to different extents [Fig. 1A (plateau values) and B (y intercepts)]. Presumably, they are able to displace bound peptides to different extents. To further characterize the binding affinity of these copolymers, competitive binding assays were carried out with biotinylated Cop 1 and unlabeled inhibitors [Cop 1, poly(Y,A,K), poly(Y,E,A), poly(E,A,K), poly(Y,E,K), MBP 84–102 and HA 306–318 peptide] (Fig. 2). The binding of biotinylated Cop 1 to detergent-soluble HLA-DR1 and -DR4 molecules was efficiently inhibited by unlabeled poly(Y,E,A), poly(Y,A,K), HA 306–318 peptide and Cop 1, but >10-fold less by poly(Y,E,K) and poorly by poly(E,A,K), as expressed by the 50% inhibitory dose (IC_{50}) (Fig. 2 and Table 1). A similar inhibition pattern was obtained for HLA-DR2 (Table 1). These results show that the copolymers of three amino acids, in particular poly(Y,A,K) and poly(Y,E,A), bind to class II MHC molecules with an affinity range similar to that of antigenic peptides and of Cop 1 for which they are effective competitors. Notably, MBP 84–102 was a very poor inhibitor of the binding of Cop 1 to HLA-DR2.

Effect of superantigens on the binding of copolymers to HLA-DR molecules

Bacterial superantigens staphylococcal enterotoxin (SE) A, SEB and TSST-1 inhibited Cop 1 binding to purified HLA-DR molecules only at very high concentrations (15). To examine the effect of these superantigens on the binding of copolymers of three amino acids to purified HLA-DR1, -DR2 and -DR4 molecules, competitive binding assays were carried out with unlabeled SEA, SEB and TSST-1. Poly(Y,A,K) binding to HLA-DR1, -DR2 and -DR4 was only inhibited by the superantigens at very high molar ratios of superantigen:poly(Y,A,K) (50:1) (Fig. 3A), whereas the binding of poly(Y,E,A) and poly(E,A,K), which bound with lower affinity, was inhibited more significantly by the superantigens (Fig. 3B and C).

Cross-reactivity of Cop 1-derived copolymers with Cop 1

To determine whether poly(Y,A,K), poly(Y,E,A), poly(E,A,K) or poly(Y,E,K) cross-react with Cop 1 at the level of T cell recognition, Cop 1-specific mouse and human T cell lines were tested for proliferation and IL-4 secretion in response to these copolymers. The six different lines listed were quite heterogeneous in their responses. However, individual mouse and human T cells cross-reacted with poly(E,A,K) and poly(Y,E,A), whereas poly(Y,A,K) was reactive with only one of the four mouse lines but with neither of the two human lines (Table 2). Poly(Y,E,K), on the other hand, did not exhibit cross-reactivity with any of the six Cop 1-specific lines (Table 2).

Discussion

Promiscuous and efficient binding of Cop 1 to intact APC of both mouse and human origin (11), as well as to purified human HLA-DR molecules (15), has previously been shown. The question whether all four amino acids that comprise Cop 1 (Y,E,A,K) are necessary for its binding was addressed by examining random synthetic copolymers compos d of only three of the amino acids that comprise Cop 1, with the molar ratio of the remaining amino acids as in Cop 1. Here, four different copolymers poly(Y,E,A), poly(Y,A,K), poly(E,A,K) and poly(Y,E,K) were observed to bind to purified HLA-DR proteins with various intensities. The binding data suggest that Y and K as well as A are important for binding f Cop 1 to the three DR haplotypes tested. Of special importance is the finding that Cop 1 demonstrated high binding activity to HLA-DR2, the MHC protein that is expressed by >60% of the general MS population. The same three amino acid copolymers also bound to living APC of both murin and human origin (unpublished data).

The cross-reactivity of the Cop 1-related copolymers with Cop 1 at the level of the T cell response is important since Cop 1-specific T cells were shown to be essential for Cop 1 therapeutic activity in both EAE (8,20,21) and MS (22). Three

Table 2. Cross-reactivity of copolymers using T cell lines and clones specific for Cop 1

Copolymer	Cross-reactivity with poly(Y,E,A,K) (Cop 1) (%)[a]									
	Murine T-cell lines and clones[b]								Human T-cell clones	
	LN-3		S-3		LN-1		S-22-5		C-14 prol.	C-52 prol.
	Prol[c]	IL-4	Prol.	IL-4	Prol.	IL-4	Prol.	IL-4		
Poly(E,A,K)	130	139	49	11	0	1	0	0	4	75
Poly(Y,E,A)	3	6	102	107	0	1	0	0	1	58
Poly(Y,A,K)	3	7	2	3	64	120	0	0	0	5
Poly(Y,E,K)	2	4	12	6	0	1	0	0	0	5

[a]Results are expressed as percent cross-reactivity obtained by the formula: [(c.p.m. in response to the tested copolymer – c.p.m. with no antigen)/(c.p.m. in response to YEAK – c.p.m. with no antig n)]×100.
[b]Cop 1-specific LN-1 and LN-3 clones were derived from lymph nodes f (SJL/J×BALB/c)F_1 mice injected with Cop 1 in complet Freund's adjuvant; S-3 line and S-22-5 clone were prepared from spleens of F_1 mice injected with Cop 1 in incompl te Freund's adjuvant.
[c]Proliferation or IL-4 secretion.

copolymers poly(Y,A,K), poly(E,A,K) and poly(Y,E,A) exhibit d cross-reactive patterns with Cop 1-specific lines and clones of both mouse and human origin. The murine T cell lines were recently characterized as T_h2 cells that cross-react with the autoantigen MBP at the level of T_h2 cytokine secretion and are capable of suppressing EAE *in vivo* (20). Since Cop 1-specific T cells were derived from peripheral blood lymphocytes of a MS patient, their ability to recognize the Cop 1-related copolymers could also be of therapeutic relevance. Of interest is the finding that poly(Y,E,K) which exhibited high binding capacity to the various MHC haplotypes did not cross-react with any of the T cell lines and clones suggesting that A is essential for T cell recognition of Cop 1. Binding to class II MHC alone may not be sufficient for Cop 1 activity *in vivo*. Additional steps involving the induction of cross-reactive T-cell tolerance or TCR antagonism may also play a role (21).

Thus, based on their binding capacity, these copolymers could be arranged in the following order: (i) binding to HLA-DR1: YEAK > YAK > YEK > YEA >> EAK; (ii) binding to HLA-DR2: YEAK > YEK > YAK > EAK > YEA; (iii) binding to HLA-DR4: YAK > YEAK >> YEA > YEK > EAK.

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Abbreviations

APC	antigen-presenting cell
Cop 1	poly(Y,E,A,K) (copolymer 1)
EAE	experimental autoimmune encephalomyelitis
HA	influenza virus hemagglutinin
MBP	myelin basic protein
MOG	myelin oligodendrocyte glycoprotein
MS	multiple sclerosis
PLP	proteolipid protein
SE	staphylococcal enterotoxin

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